X-RAY EXPERIMENTS WITH MOLGULA MANHATTENSIS: ADULT SENSITIVITY AND INDUCED ZYGOTIC LETHALITY

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Sessile organisms which pump large quantities of sea water through their bodies suggest themselves as ideal material in which to study biological effects of radio-active contaminants. Because of their relationship to the chordates, ascidians have particular attraction. Furthermore, many are functional hermaphrodites providing both sperm and eggs for simultaneous irradiation. *Molgula manhattensis* has the additional advantage of self-fertility so that crosses using gametes from the same individual can be made, as well as outcrosses.

In the absence of radiobiological information on Molgula, the present x-ray experiments were performed rather than employing isotopes with their more difficult dosimetry. Isotope experiments may be performed in the future after a more complete knowledge of what may be expected from external radiations is at hand.

MATERIALS AND METHODS

Preliminary experiments were performed during the summer of 1955 with the assistance of Robert L. Sullivan. Specimens of Molgula were collected for us by personnel of the M. B. L. Supply Department. Irradiation was delivered by the M. B. L. generator in "A" position at 6000 r per minute (187 KV; 28 ma; filtration $\equiv 0.2$ mm. Cu). To achieve massive doses without overheating, the animals were immersed in sea water within an inner chamber, and crushed ice was packed into the space between it and the outer wall of the plastic container. After irradiation, animals were kept in individual glass jars (50 × 80 mm.) in running sea water.

Failure to contract in response to prodding with a blunt instrument was the criterion for death. An additional check on presumptive death was obtained by holding such animals until post-mortem flaccidity and degeneration became obvious. The results indicated that adults would be adequately resistant to whole-body irradiation which would allow the full range of dosages required for a genetic experiment on induced dominant lethality.

In 1956 animals of adult size were obtained both through the M. B. L. Supply Department and by personal collection. The latter procedure seemed necessary when it was discovered that masses of Molgula held in the Eel Pond "live-cars" and in laboratory aquaria did not consistently exhibit gonads with mature gametes. The adequacy of the supply of eggs and sperm can be determined by examination with a low power microscope, usually after peeling off the test, always after removal of a portion of the mantle.

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Adults were irradiated in position "B" at 2500 r per minute, again using the Woods Hole machine, the Coolidge tubes this time not as close to the animals.

Eggs were obtained by suction applied to a capillary pipette inserted into the lumen of an ovary. They were expelled into a stender dish of sea water. Immediately after withdrawing eggs from both ovaries, sperm suspensions were prepared by macerating a portion of each testis in a separate dish of sea water (3–5 cc.).

Eggs from each ovary were divided into three groups: (1) a control as a check against accidental fertilization during removal, (2) a sample which was fertilized with sperm from the adjacent testis, and (3) a sample which was cross-fertilized with sperm from another Molgula. Sixty-millimeter flat stender dishes, containing one inch of sea water, were used. They were placed on the sea table with their bases in running water. Fifteen hours later, tadpole development was scored using a stereoscopic dissecting-type (48 ×) microscope. Immediately after completion of these observations, the degree of cleavage for 100 objects was scored using a compound microscope (16 mm. objective, 10 × ocular).

During July and the first half of August, pair-mating experiments were set up daily to obtain simultaneous data (a) for eggs from an irradiated animal fertilized by sperm from an untreated one, (b) for the reciprocal cross of treated sperm to untreated eggs, (c) from selfing the irradiated animal, and (d) selfing the non-irradiated member of the pair. The goal was to obtain data from at least five pairs of crosses for each of five chosen doses.

T. H. Morgan's 1942 paper led us to expect a technical difference between the two ovaries in difficulty of removing unfertilized eggs. Therefore we kept separate controls and made separate crosses for each side of each animal. However, upon analysis of results, no significant differences between sides were demonstrable and the data for the two sides of each animal serve merely as replications.

From August 13 through August 18 the daily plan of experiment was modified. On each day, five or more adult animals were simultaneously exposed to one of the five chosen doses. The gametes obtained from five treated animals were mixed to provide outcross data when both sperm and eggs were treated. At least five samples (averaging 800 objects/sample) were scored for each mass fertilization. On August 15 a mass outcross control was obtained.

From August 20 through August 24, five animals were treated simultaneously at each of the five doses and selfed to obtain more information about the variability between material from different treated animals.

From August 27 to September 1, miscellaneous experiments were set up: crosses of three different animals, each given a different dose, to a single untreated animal, and selfing crosses in which several doses were investigated each day.

Results

Lethality of adults

An exploratory experiment indicated that the critical dose of x-ray for the adult organism lay between 36,000 r and 60,000 r. This involved observations on 72 sea-squirts, 8 of which were controls, along with 8 samples of 8 animals given doses graded between 1000 r and 120,000 r.

Two subsequent experiments of 40 each were set up with controls and samples irradiated at the following doses: 36,000 r, 42,000 r, 48,000 r, 54,000 r and

60,000 r. In both the control and 36,000 r groups, individual animals were still alive more than a month after the date of irradiation. Also, although most of the animals given 42,000 r died during the first week, one lived almost a month. At higher doses no animals survived the fourth day, with the average time of death 2.4 days after treatment. This places the lethal dose between 42 and 48 kiloroentgens. This is considerably less than the radiation required to kill adult insects (Sullivan and Grosch, 1953), brine shrimp (Grosch and Erdman, 1955) and vegetative microorganisms (Bacq and Alexander, 1955). On the other hand, such amounts of radiation are many times that required to kill mammals.

Induced zygotic lethality

A summary of the relative proportion of tadpoles obtained among the ova and zygotes studied is given in Figure 1. Consistently fewer swimming tadpoles emerged than developed to tadpole morphology. Furthermore, curves for data from irradiated eggs tend to lie below those representing sperm. This trend becomes statistically significant at higher doses and it should be re-emphasized that the data come from paired matings.

With self-fertilization, involving sperm and eggs from irradiated sea-squirts, the developmental yield is strikingly decreased (solid line, "Both Treated"). Along with the curve for selfing data, results from mass outcross experiments are shown by the broken line in Figure 1. With one exception, points obtained by calculating the mean are nearly identical whether selfing or outcrossing has been the procedure, provided both sperm and eggs are from irradiated animals. The standard errors omitted from the outcross curve in order not to further complicate Figure 1 are less than 2%.

An additional group of data obtained from selfing five animals irradiated simultaneously at each dose also gives a curve similar to the solid line at the lower doses and identical with it at the three higher doses. Therefore it is not shown here.

Since it has been corroborated by three separate sets of experiments, the curve above 5,000 r is a good representation of expectation when both sperm and eggs come from gonads irradiated *in situ*. Furthermore, this "Both-Treated" curve is predictable on the basis of one of the laws of probability: when two events are independent, the probability that both will occur simultaneously is the product of their separate probabilities. Thus, multiplying survival when sperm are x-rayed by survival when eggs are x-rayed we obtain the following:

$$.33 \times .075 = .0248 = 2.5\% \text{ for } 20,000 \text{ r}$$
$$.34 \times .185 = .0629 = 6.3\% \text{ for } 15,000 \text{ r}$$
$$.45 \times .43 = .1935 = 19.4\% \text{ for } 10,000 \text{ r}$$
$$.545 \times .51 = .2779 = 27.8\% \text{ for } 5,000 \text{ r}$$

These values calculated from Figure 1 data for the five higher doses are all within the range of one standard error from the "Both-Treated" curve shown in Figure 1. The theoretical value for 1000 r, 24.6% (.53 × .465), is lower than the "selfing" value obtained in pair experiments but falls within the range found in outcross experiments when both sperm and eggs are from treated animals.
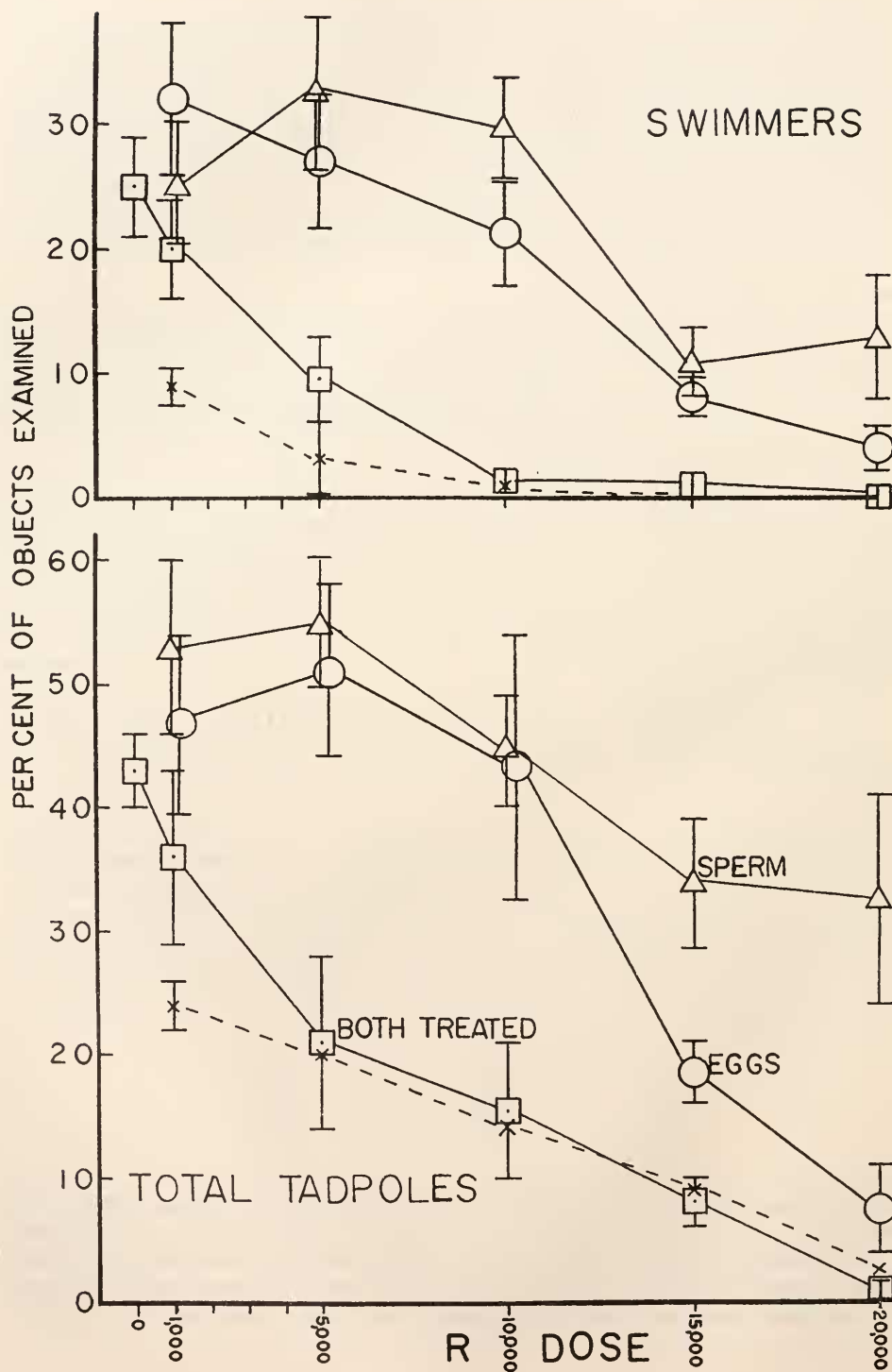
TABLE I

Development in the residuum of eggs and zygotes, tadpoles having been counted. The scoring was done on 100 objects in each case. Values are given in per cent, representing failure to develop to tadpole morphology (means and standard errors)

X-ray dose in r	Both gametes treated			Sperm treated	Eggs treated
	Mass outcross	Self-cross 5 simultaneously	Self-cross pair experiments		
0	99.5 ± 0.5		45.8 ± 11.9		
1,000	84.3 ± 3.3	76.5 ± 0.5	54.2 ± 10.8	74.6 ± 6.3	63.8 ± 7.9
5,000	90.5 ± 2.0	72.2 ± 7.4	35.4 ± 12.9	55.4 ± 3.4	57.4 ± 9.8
10,000	89.9 ± 4.7	93.6 ± 2.9	59.2 ± 12.2	65.2 ± 5.6	64.8 ± 12.6
15,000	91.2 ± 1.2	79.8 ± 12.7	70.5 ± 8.4	65.9 ± 5.3	75.0 ± 6.8
20,000	86.1 ± 1.6		36.2 ± 10.2	76.0 ± 5.0	50.4 ± 11.7

No attempt was made to obtain a quantitative record of morphological aberration. However, as an indication of developmental difficulty, thickened, bent and misshapen tails were typical for tadpoles in experiments above 10,000 r.

Cleavage data

In percentages, Table I presents a summary of results when objects other than tadpoles were scored. These are the averages from five or more experiments, in each of which 100 objects were carefully examined. Because of the time required and technical difficulty it is not feasible to examine all of the residuum. In order to understand Table I it must be realized that when few tadpoles develop from a group of eggs there are many undeveloped forms, and vice versa. Accordingly, since the amount of residuum varies, similar percentages with different doses may actually reflect differences. A conversion of the tabulated percentage values into numerical values, such as those plotted in Figure 2, helps to visualize the situation. The average total of eggs per stender dish, 800, is used as a common basis for presentation. The pertinent percentage of tadpoles (see Fig. 1) is subtracted. The applicable cleavage percentage (Table I) of the remainder gives the relative number of embryos plotted in Figure 2.

All embryos considered in Table I and Figure 2 appeared to be in the late gastrula or neurula stages, although at the two highest doses, structure was very disorganized. Presumably if embryos were able to begin development they could continue to such stages before facing an insurmountable developmental crisis. Extremely few embryos were found halted in an early cleavage stage. During the whole summer, when nearly 150,000 examinations were made, only 18 early cleavage types were seen in selfing experiments, and 17 in outcrosses—exceptional individuals making up only 0.02%.

As might be expected, the number of gastrulae and neurulae increased as the number of tadpoles decreased at higher doses. Although this general trend is clear, unidentified sources of variability complicate the picture in selfing and pair-

FIGURE 1. The relative proportions of tadpoles developing from gametes obtained from adult specimens of Molgula after irradiation. Results are contrasted when either or both types of gametes come from x-rayed parents.
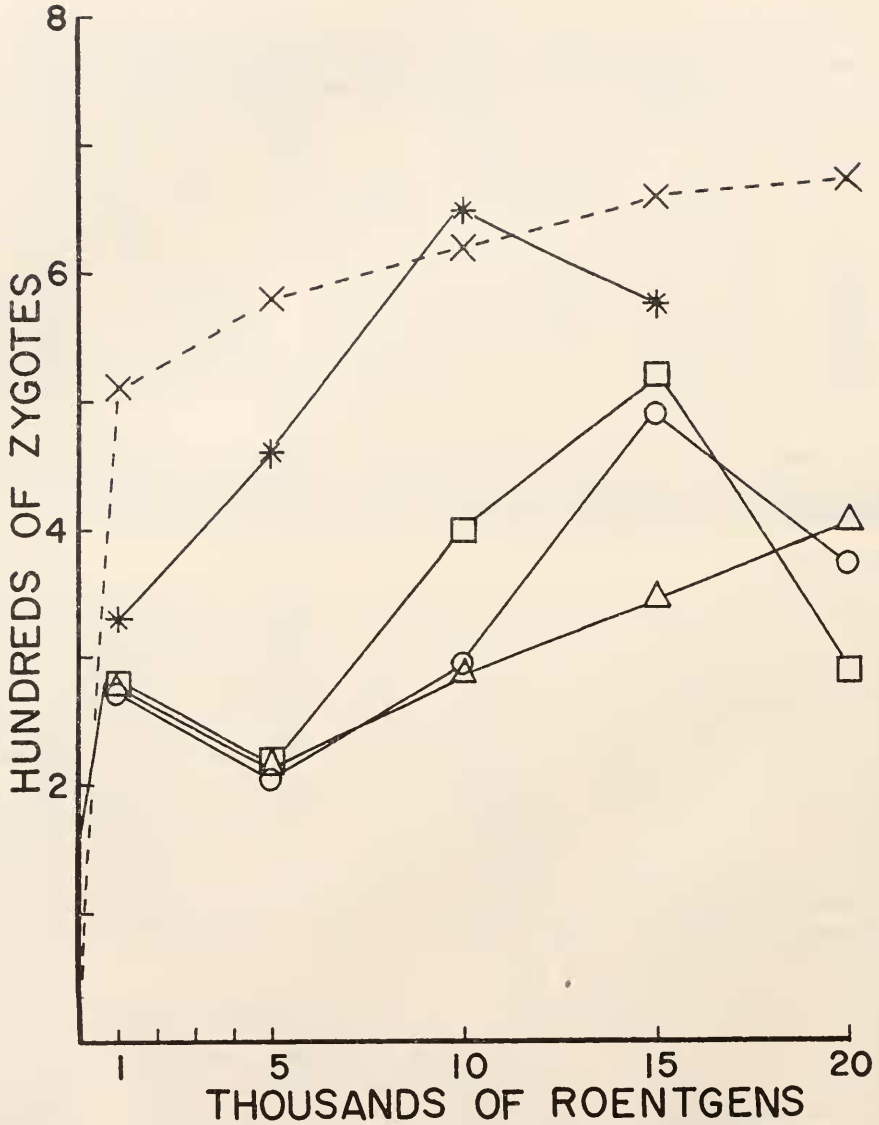
FIGURE 2. Post-cleavage zygotes which failed to develop to the tadpole stage. Results have been put on a common basis by calculations from the average total of eggs per sample, 800. Triangles indicate that the sperm were from treated animals; circles, eggs. Squares represent selfed eggs and sperm from treated animals in pair experiments; asterisks, data from 5 irradiated animals selfed on the same day. X's indicate outcross data, both parents x-rayed. Squares, circles and triangles represent results from pair experiments.

mating experiments. When plotted as in Figure 2, zigzag lines are obtained. Furthermore, in the latter experiments, results when both gametes came from treated parents are not greatly different from those obtained when only one of the

types of gametes has been exposed to radiation. Indeed, even after 5000 r, all three pair mating values were nearly identical. Therefore some general aspect of fertilization common to the experiments should be sought. The higher average values obtained after simultaneously selfing five animals given the identical dose of radiation are consistent with such a view.

Discussion

Relative radiosensitivity of sperm and eggs

In Molgula, relative radiosensitivity of eggs instead of sperm is an interesting parallel to Rugh's (1953) similar findings with the clam Spisula, although his crosses involved gametes which were irradiated after extraction from the parent. Such results are not expected, either on the basis of Henshaw's type of delayed cell division or that of chromosomal-gene effects. Sperm have been found more sensitive on either count.

Mavor and De Forest (1924), who irradiated Arbacia gametes, found that samples scored two days later showed development ranging from gastrula stages to well-formed plutei. The retardation in development was greatest in those larvae developed from x-rayed sperm and increased with dose. Subsequently, Henshaw (1940) explained this phenomenon by a demonstration that radiation-delayed first cleavage is reflected in later development. Furthermore, the apparent difference in sensitivity was due to partial recovery occurring in the eggs prior to fertilization.

Judging from insect experiments, dominant lethals are more readily induced in sperm. In fact, P. W. Whiting (1938) found that at dosages of about 10,000 r, practically every Habrobracon sperm contained at least one dominant lethal. Certain of his experiments more closely resemble the conditions of the present experiment than any other investigations we have been able to find in the literature. In these experiments, female wasps mated before treatment contained both sperm and eggs when irradiated. Comparisons with data from females mated after irradiation, and with parthenogenetic data, indicated dominant lethals to be more readily induced in sperm than even recessive lethals in the eggs. In a definitive Habrobracon paper, Heidenthal (1945) constructed the dominant lethal mutation curve for doses up to the asymptote and demonstrated that those secured for Drosophila (Sonnenblick, 1940; Demerec and Fano, 1944) were quite similar. An even steeper curve has been reported for Mellitobia (Kerschner, 1946). Muller and the Valencias (1949) have since presented Drosophila data which indicate that presumptive deficiencies are far less abundant if eggs are irradiated.

Nucleic acid or its cycle is implicated in both the Arbacia cleavage delay experiments and the dominant lethal experiments with insects. Although usually discussed separately, it seems possible that both types of damage may be reflected in the results obtained by scoring development at a specified time. However, it has been shown that neither phenomenon completely explains the present results. Perhaps a third and somewhat different aspect of cell division—other than chromosomal —is involved. The material may need to be considered from the standpoint of the general physiologist who studies the stimulus for initiating the process of cell division (Heilbrunn, 1955; Heilbrunn and Wilson, 1955; Rieser, 1955).

Especially provocative is an earlier Arbacia paper (Heilbrunn and Young, 1935), which shows that irradiation in the presence of ovarian tissue produced a

considerably greater division delay than irradiation of eggs in sea water alone or in concentrated suspension. At least, sperm inactivation can be ruled out. This requires doses in excess of 100,000 r in marine forms as well as insects (Maxwell, 1938; Henshaw, 1940; Rugh, 1953).

Self-sterility

Another aspect of Molgula investigations which may turn out to be a problem in physiology rather than genetics is fertility-sterility. A range from perfect self-fertility to absolute self-sterility has attracted geneticists to ascidians from the early days of genetics research (Morgan, 1904). However, although T. H. Morgan himself devoted considerable attention to the problem, including experiments with Molgula (1942), neither the genetic basis nor the physiological mechanisms have been completely elucidated.

Observations during the present experiments revise the Molgula picture. Self-incompatibility is not as extensive as previously believed, provided (1) that organisms with undeveloped or senile (degenerate?) gonads are not used, and (2) that no strong chemical cleaning solution or detergent is employed in cleaning glassware. In all our experiments in which these criteria were met, Molgula adults were self-fertile. The influence of a chemical agent was demonstrated dramatically one day when Alcanox had been used on the glassware. In spite of repeated water washings, as is the standard procedure in analytical chemistry, and over-night drying, no development occurred in selfing and only about 5% development in outcrosses. Ordinarily in outcrosses by far the great majority of eggs are fertilized and cleave. Although no details are available, at least one of Morgan's Molgula experiments bears a resemblance to this exceptional one of ours. He recorded a case in which no eggs selfed and only two out of a large number of eggs cleaved when cross-fertilized. Perhaps hot water is the only safe cleaning agent, although the junior author feels that dilute HCl rinses do much to offset the Alcanox type of hazard.

SUMMARY

1. The lethal dose of x-rays for adult specimens of Molgula is placed in the neighborhood of 45,000 r (delivered at a rate of 6000 r/minute).

2. Radiation damage to gametes from irradiated adults can be measured in terms of tadpoles, unhatched or swimming. Eggs proved more sensitive than sperm. Curves when both gametes come from irradiated parents were similar no matter how obtained, pair matings or group matings, selfed or outcrossed. In the latter curves, 10,000 r is about the limit for swimmers, and 20,000 r for tadpole development (rate, 2500 r/minute).

3. The cleavage score for 100 objects residual to developed tadpoles did not provide a regular, clear-cut picture of radiation damage. It is suspected that uninvestigated features of fertilization physiology cloud the issue.

4. Self-incompatibility in Molgula is not as extensive as previously believed. The condition of the gonads must be considered and chemical cleaning solutions should be avoided.

5. It is concluded that the physiology of spindle formation rather than that of nucleination or chromosomal continuity may be a most important aspect of results like the present.

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